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Adaptation to Multiday Ozone Exposure is Associated with a Sustained Increase of Bronchoalveolar Uric Acid

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The phenomenon of ozone tolerance is described, but the underlying mechanisms remain unknown. We tested whether adaptation to multiday ozone exposure was related to an upregulated pulmonary antioxidant defence. Six calves were exposed to 0.75 ppm ozone, 12 h day^{-1} for seven consecutive days. Pulmonary function tests and bronchoalveolar lavage (BAL) were performed before, after the first (D_1), third (D_3) and seventh (D_7) exposure. Differential cell count, total proteins, 8-epi-$PGF_{2\alpha}$, glutathione and uric acid were determined in BAL. Dynamic lung compliance and arterial oxygen tension were significantly decreased and lung oedema impaired pulmonary function on D_1. By repeating ozone exposures, progressive functional adaptation occurred. Ozone induced a significant increase of BAL neutrophil percentage on D_1. On D_3 and D_7, neutrophil percentage was progressively decreased, but remained significantly elevated. BAL total proteins were significantly increased on D_1 and decreased progressively until D_7. 8-Epi-$PGF_{2\alpha}$ was significantly increased on D_1 and was returned to baseline on D_3 and D_7, whilst glutathione significantly increased on D_3 and returned to baseline on D_7. Uric acid was increased ten-fold on D_1. On D_3, uric acid was increased six-fold and was persistently elevated at D_7. This study suggests that ozone adaptation of functional and inflammatory variables is accompanied with sustained BAL uric acid elevation.

Keywords: Ozone; Lung function; Airway inflammation; Oxidative stress; Adaptation

INTRODUCTION

Ozone (O_3) is an atmospheric pollutant and represents the most important environmental oxidant, submitting plants, animals and human beings to oxidative stress.[1] Over the last three decades, many animal and human studies have focused especially on pathophysiological pulmonary effects of this oxidant gas. The health effects of acute O_3 exposure have been assessed in numerous controlled exposure studies demonstrating three types of lung response to O_3: airway inflammation, pulmonary function decrease and airway hyperreactivity.[2] However, Stokinger described for the first time in 1956 the phenomenon of O_3 tolerance or O_3 adaptation, which occurs after multiple O_3 exposures and which may mask or attenuate the functional O_3 responses.[3] Since then, the functional and inflammatory responses to single and repeated O_3 challenges have been widely investigated (for review see Refs. [2,4–6]). Nevertheless, the mechanisms underlying adaptation remain unknown. Recent studies suggested that the pulmonary antioxidant defence mechanism may be involved.[7–9] The respiratory tract lining fluids (RLTF) form an interface between the underlying respiratory tract epithelial cells and the external environment. It thus constitutes a "first line of defence" against inhaled oxidants, such as O_3.[8] The RTLF contains various antioxidants that may provide protection from oxidants. Uric acid, glutathione, ascorbic acid, Vitamin E, thiols and mucins are the most important RTLF antioxidant components[1] and their concentrations in RTLF have been shown to be modified by O_3.[9,10] As the RTLF components are mediating the toxic action of O_3 to the underlying

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epithelial cells, the RTLF encloses also oxidation products such as lipid hydroperoxides, ozonides, aldehydes, etc., which are reflecting oxidative damage.[1] Moreover, these by-products may play an important role by promoting lung inflammation.[11,12] Isoprostanes, and especially 8-epi-$PGF_{2\alpha}$, are products of membrane lipid peroxidation and have been shown to be not only reliable markers but also implicated in pulmonary oxidative stress.[13,14] In fact, isoprostanes are cyclooxygenase-independent prostaglandins whose synthesis is enhanced by radical-catalysed lipid peroxidations. Pulmonary 8-epi-$PGF_{2\alpha}$ concentrations have been shown to be increased by acute and chronic lung disease[15,16] and by single O_3 exposure.[17] Moreover, the fact that 8-epi-$PGF_{2\alpha}$ is an *in vitro* and *in vivo* bronchoconstrictor in animals and humans confers additional importance to this oxidative damage marker.[18,19]

Recent O_3 exposure studies performed in humans focused interest on three key points and the relations existing between these hallmarks, namely lung function, airway inflammation and pulmonary antioxidant status,[7,20,21] but the investigations were limited by the impracticability of repeated and invasive experimental procedures in human patients, e.g. airway endoscopy, bronchoalveolar lavage (BAL), etc. In the study presented here, the bovine species was chosen as animal model because of its easy handling, the possibility of performing extensive pulmonary function tests (PFT) and the facility to repeat daily airway endoscopy and BAL.

The current study sought to describe a standardised animal model of multiday O_3 exposure challenge allowing repeated and simultaneous assessment of the functional, inflammatory and antioxidant pulmonary response. The functional respiratory response to repeated O_3 exposures was assessed by multiple PFT, whilst bronchoalveolar proteins and cell populations were used as markers of airway inflammation. Simultaneously, the impact of O_3 on the RTLF antioxidant defence system was examined by determination of isoprostane (8-epi-$PGF_{2\alpha}$) as an oxidative damage marker and glutathione and uric acid as antioxidant markers. It was hypothesised that the O_3 tolerance phenomenon was correlated with an upregulated antioxidant defence system.

MATERIALS AND METHODS

Animals

Six Holstein Friesian 4-month-old calves (mean bodyweight±standard deviation, 133.5 ± 15.7 kg), free of airway diseases were used for this study. The animals were selected on base of their clinical examination, PFT and airway endoscopy including cytological analysis of BAL. Once admitted for the protocol, the calves were housed in individual boxes in a barn connected with the O_3 generating system, allowing identical management and simultaneous O_3 exposure. The study was approved by the Animal Ethics Committee of the University of Liege.

Study Design

All calves underwent simultaneously for 12 h exposure to 0.75 ppm O_3 during seven consecutive nights. Clinical examination, PFT and endoscopy of the airways were performed 2 h after cessation of O_3 exposure.

On Day 0 of the study, clinical examination, arterial blood gas analysis, venous lactate determination and measurement of baseline ventilatory mechanics followed by airway endoscopy and BAL were performed. Six hours after endoscopy, the first O_3 exposure began. On Day 1, Day 3 and Day 7 the same measurements as on Day 0 were performed.

Ozone Exposure System

The exposures took place in a barn (94.5 m^3) in which the calves were housed in individual boxes. The barn was tightly closed during exposures, and ambient air was continuously drawn through the chamber with a ventilation rate of 40 $m^3 h^{-1}$. Homogenous ventilation and air circulation of the barn were previously checked by smoke distribution. Ozone was generated from ambient air by a Compact Industrial Ozone Generator TOG C2 (Ozonia Triogen Ltd, Glasgow, Scotland) and analysed by an ultraviolet light photometer (Portacel Ozone Monitor LC 400, PCI Ozone and Control Systems, New Jersey, USA). The O_3 concentration was measured every 20 s and was displayed continuously. The generator was calibrated with a standard O_3 monitor (BMT Messtechnik 931 UV, BMT Messtechnik GmBH, Berlin, Germany). The target O_3 concentration of 0.75 ppm was maintained within deviations of less than 5%. Temperature (22.5 ± 1.2°C) and relative humidity (60 ± 2%) of the exposure barn were measured at the beginning and the end of each O_3 challenge. No significant variations of room temperature or humidity were measured, neither during nor between O_3 exposures.

Clinical Examination

Prior to the PFT, the calves were shortly examined. Special attention was focused on psyche and on presence of nasal and buccal foam, indicating lung oedema. Furthermore, each calve was auscultated in order to detect subclinical oedema.

Pulmonary Function Tests

PFT included assessment of ventilatory mechanics and arterial blood gas tension analysis. As plasma lactate has been demonstrated to be a reliable prognostic indicator in bovine pulmonary disorders,[22] its determination was also performed.

Ventilatory mechanics measurements were performed for 2 min when calves were breathing normally at rest by use of the oesophageal balloon catheter technique.[23] Intrapleural pressure was measured by means of an oesophageal balloon catheter made from a condom sealed over the end of a polyethylene catheter (2 mm ID, 3 mm OD, 120 cm long, VEL, Leuven, Belgium) positioned with its tip in the middle thoracic oesophagus and connected to a pressure transducer (Valydine M1-45, Valydine Engineering, Northridge, CA, USA). An airtight facemask covered calves' nostrils and mouth. A Fleisch pneumotachograph Nr. 3 measuring respiratory airflow was mounted on the facemask and was coupled by two identical catheters (4 mm ID, 6 mm OD, 120 cm long, VEL, Leuven, Belgium) to a differential pressure transducer (Valydine DP45-18, Valydine Engineering, Northridge, CA, USA). Respiratory airflow and oesophageal pressure were simultaneously measured and respiratory rate (RR), total pulmonary resistance (R_L), dynamic compliance (C_{dyn}) and maximal pleural pressure changes (MaxΔPpl) were continuously calculated and recorded by a computerised system (Po-Ne-Mah, Gould Instrument Systems, Valley View, OH, USA). Volume and pressure calibrations were performed with a 2 l pump (Medisoft, Dinant, Belgium) and a water manometer, respectively. Further technical details are provided elsewhere.[24]

Arterial blood was withdrawn anaerobically by puncture of *Ar. Carotidis communis* and analysed, after correction for body temperature, for partial pressure in oxygen (PaO_2) and carbon dioxide ($PaCO_2$) (AVL 995, VEL, Leuven, Belgium).

Plasma lactate (L_A) was analysed in venous blood obtained by jugular vein puncture by a lactate analyzer (Accusport®, Boehringer Mannheim, Mannheim, Germany).[22]

Bronchoscopy and Lavage Procedure

BAL was performed on the sedated calves (xylazine 0.2 mg kg^{-1} bw iv, Xyl-M® 2%, VMD, Berendonk, Belgium) using a 250 cm fibreoptic endoscope (Pentax, Breda, Netherlands) wedged in the bronchi, and by instilling *in situ* 60 ml of sterile saline (NaCl 0.9%) heated at 37°C. This small lavage volume was intentionally used in order to prevent further lung function impairment after O_3 exposure on Day 1. The BAL was considered to be successful when alveolar surfactant and more than 60% of the instilled fluid were recovered. Recovery of BAL fluid ranged from 60 to 75% of the saline fluid instilled. Recovered BAL fluid was immediately cooled on ice and processed for definitive storage until analysis. The calves' sedation was reversed by intravenous atipamezole injection (0.2 ml per calf, Antisedan®, Pfizer Animal Health, Brussels, Belgium). Care was taken to perform repeated BAL procedures alternatively in both lung sides and in different airway segments.

Bronchoalveolar Lavage Processing

One portion of BAL fluid for total protein determination and uric acid analysis was centrifuged for 5 min at 2500*g* and 4°C. Supernatant was stored at −80°C until analysis. For glutathione analysis, methanol was added to BAL fluid (0.7 ml ml^{-1} BAL fluid) which was centrifuged for 2 min at 13,000*g* and 4°C. Supernatant was withdrawn and stored in liquid nitrogen. For 8-epi-$PGF_{2\alpha}$, aliquots of 5 ml untreated BAL fluid were stored at −80°C. The remaining BAL fluid was centrifuged (4 min, 500*g*) and the cell pellet was prepared for cytological analysis. Differential cell count of BAL fluid was performed on Diff Quick stained preparations.

Analysis of BAL

Total protein concentration in BAL was photospectrometrically determined (Total Protein Ultra Sensitive, Eli Tech Diagnostics, Sees, France). 8-Epi-$PGF_{2\alpha}$ in BAL was purified and concentrated before being analysed by an enzyme immuno-assay kit (Cayman, Ann Arbor, MI, USA). Briefly, BAL samples were defrosted and pH adjusted to 2–2.5 with 2 normal hydrochloric acid (HCL 2N). BAL was centrifuged for 10 min at 200*g* and 4°C. Two ml of supernatant were run through a C_{18} 1 ml 100 mg^{-1} column (Bond Elut, Varian, Harbor City, CA, USA). Column was rinsed with 1 ml water and 1 ml hexane. Elution was performed with 2 ml (4 × 500 µl) ethyl-acetate/methanol (95:5, v/v). Eluate was vacuum dried and restituted with 500 µl of buffer provided by the kit manufacturer. The kit was consequently used according to manufacturer's instructions. Uric acid was analysed by high performance liquid chromatography (HPLC) according to the method of Grootveld and Halliwell.[25] Reduced glutathione (GSH) and oxidised glutathione disulfide (GSSG) in BAL were measured by use of HPLC with electrochemical detection according to the method described by Smith and co-workers.[26]

Preliminary studies performed on calves have shown that the recovery of BAL fluid and the proportion of epithelial lining fluid (ELF) in BAL calculated by the urea method are not significantly modified by O_3 exposure (unpublished data), as it is described in humans.[20] This is why the

concentrations of compounds analysed in BAL were expressed per ml of BAL rather than per ml of ELF.

Statistical Analysis

Data are presented as mean±standard deviation (SD). Data were firstly analysed by an ANOVA for repeated measures (parametric data) or by a Friedman test (non-parametric data). Significant differences were further analysed either by a Student's paired t-test (parametric data) or by a Wilcoxon's sign ranks matched-pairs test (nonparametric data). Relationships between two variables were analysed by use of linear regression. The level for statistical significance was set at 0.05.

RESULTS

Clinical Examination

On Day 0, all subjects were clinically healthy. After the first O_3 exposure, the clinical state of all calves was very similar, confirming that all animals housed in the barn underwent the same ozone challenge. The calves were prostrate and suffering from severe lung oedema, as shown by the presence of nasal and buccal foam. Pulmonary auscultation confirmed this finding. On Day 3, the calves were less prostrate and clinical signs of lung oedema were decreased but still detectable by auscultation. On Day 7, all animals showed normal behaviour and clinical signs of lung oedema were no longer detectable.

Pulmonary Function Tests

Ventilatory Mechanics

Among the recorded variables, dynamic lung compliance (C_{dyn}) underwent the most striking variations (Fig. 1A). Measurements indicated a drastic and significant decrease in C_{dyn} after the first exposure ($P < 0.05$). Dynamic lung compliance values at Day 3 were increased but were still significantly lower than baseline value ($P < 0.05$). At Day 7, C_{dyn} values remained slightly lower than at pre-exposure. Total pulmonary resistance (R_L) did not change significantly throughout the protocol. Maximal variation of pleural pressure (MaxΔPpl) was significantly increased after the first exposure (2.40 ± 0.38 versus 1.43 ± 0.64 kPa) ($P < 0.05$), but reached baseline values during the following days. RR was increased in a non-significant manner on Days 1 and 3. At Day 7, RR was significantly decreased as compared to pre-exposure (35.2 ± 6.9 versus 41.5 ± 3.4) ($P < 0.05$).

Arterial Blood Gas Analyses and Plasma Lactate

Partial arterial oxygen pressures were significantly decreased at Day 1 and Day 3 ($P < 0.05$). On Day 7, PaO_2 values increased progressively, but nevertheless remained lower than at pre-exposure (Fig. 1B). $PaCO_2$ values were significantly lower on Day 1 (41.0 ± 3.8 versus 45.7 ± 2.7 mm Hg) ($P < 0.05$). During the following days, CO_2 tension values were similar to those of baseline. Venous plasma lactate values were significantly increased at Day 1 ($P < 0.05$) and decreased during the following days (Fig. 1C).

BAL Inflammatory Markers

Differential Cell Counts in BAL

Neutrophils were significantly increased at Days 1, 3 and 7 ($P < 0.05$), even though there was a tendency to decrease after Day 1 (Figs. 2A and 4). As a consequence of the sharp neutrophil increase, macrophage and lymphocyte percentages were significantly decreased on Days 1 and 3, and on

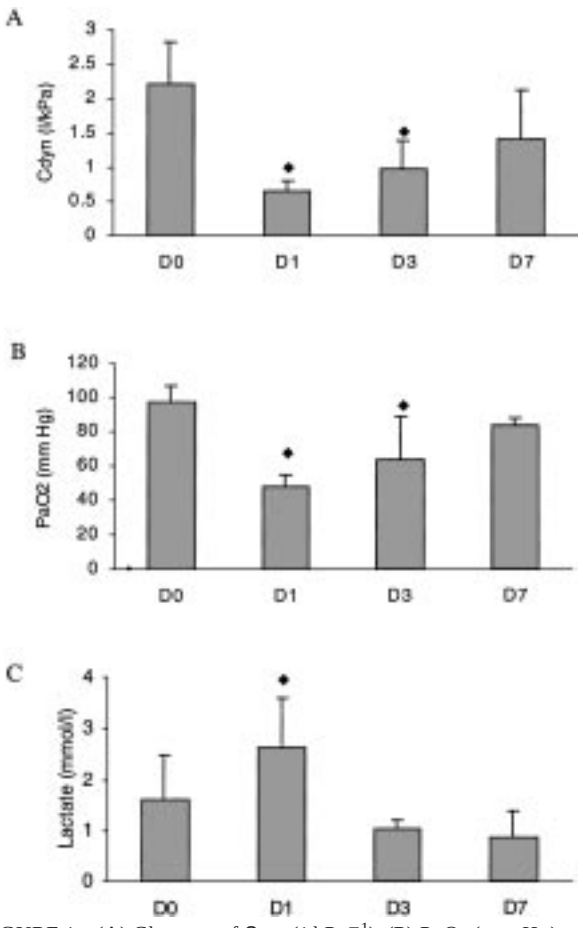

FIGURE 1 (A) Changes of C_{dyn} ($1\,kPa^{-1}$), (B) PaO_2 (mm Hg) and (C) plasma lactate ($mmol\,l^{-1}$) before (D_0), and after one (D_1), three (D_3) and seven (D_7) O_3 exposures (0.75 ppm, 12 h day^{-1}). Data are presented as mean ± SD. ◆ Significantly different from D_0 values, with $P < 0.05$.

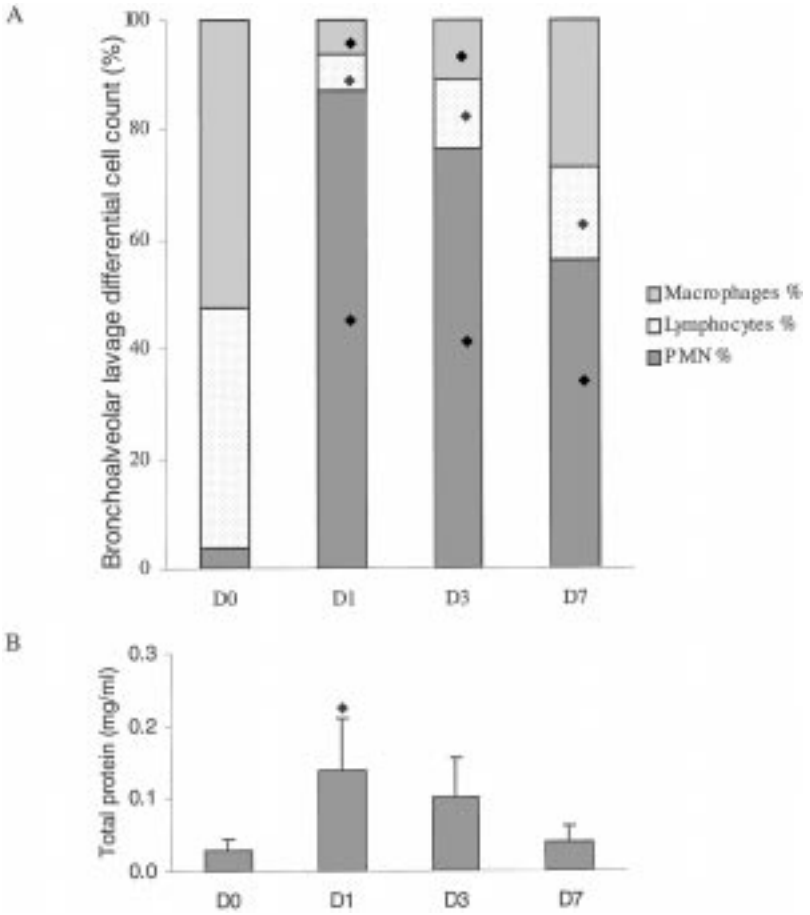

FIGURE 2 (A) Differential BAL cell count (%) before (D_0), and after one (D_1), three (D_3) and seven (D_7) O_3 exposures (0.75 ppm, 12 h day^{-1}). Data are presented as means, SD were omitted for clarity. ◆ Significantly different from respective D_0 values, with $P < 0.05$. (B) total protein concentrations (mg ml^{-1}) in BAL fluid collected before (D_0), and after one (D_1), three (D_3) and seven (D_7) O_3 exposures (0.75 ppm, 12 h day^{-1}). Data are presented as means ± SD. ◆ Significantly different from D_0 values, with $P < 0.05$.

Days 1, 3 and 7, respectively ($P < 0.05$). No eosinophils and basophils were found in BAL.

BAL Total Protein Concentration

Total protein concentrations are shown in Figs. 2B and 4. BAL total proteins were significantly increased by the first exposure ($P < 0.05$). Total protein levels decreased progressively, but remained slightly elevated as compared to baseline values.

BAL Oxidant and Antioxidant Markers

Modifications of BAL 8-epi-$PGF_{2\alpha}$ are illustrated in Figs. 3A and 4. After the first exposure, the concentrations of 8-epi-$PGF_{2\alpha}$ were significantly elevated. At Day 3, 8-epi-$PGF_{2\alpha}$ levels were similar to those at baseline. At Day 7, levels were significantly lower than at pre-exposure.

BAL GSH was unchanged after the first O_3 exposure and was significantly increased on Day 3 compared with Day 1 ($P < 0.02$) (Figs. 3B and 4). GSH levels were diminished at Day 7, but were not significantly different from Day 0. GSSG and total glutathione (TGSH = GSH + GSSG) followed a similar trend. The glutathione redox ratio was calculated as follows: GRR% = [GSSG/(GSH + GSSG)]. GRR was decreased on Days 1 and 3, but in a non-significant manner. On Day 7, GRR was slightly higher than on Day 0.

BAL uric acid increased as a result of the O_3 challenge and remained significantly higher throughout the protocol ($P < 0.05$) (Figs. 3C and 4). The maximum increase occurred following the first exposure, but at Days 3 and 7 uric acid levels remained significantly higher than baseline.

Correlation Analyses

The changes of C_{dyn} were significantly correlated with MaxΔPpl ($P = 0.0022$, $r = -0.545$). MaxΔPpl was significantly correlated with L_A ($P = 0.011$, $r = 0.595$). C_{dyn} and bronchoalveolar neutrophil percentage were highly correlated ($P < 0.0001$, $r = -0.742$). PaO_2 and neutrophil percentage were also correlated ($P < 0.0001$, $r = -0.810$). Bronchoalveolar total proteins were significantly correlated with C_{dyn} ($P = 0.103$, $r = -0.547$). MaxΔPpl ($P = 0.005$,

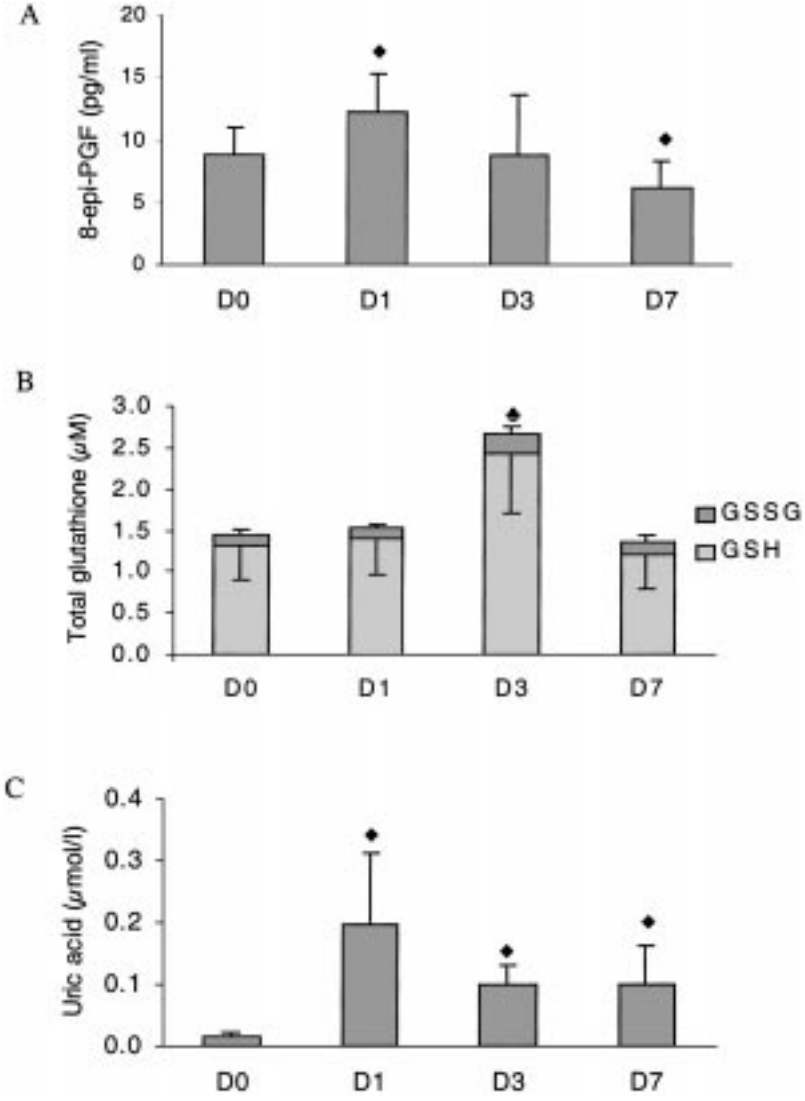

FIGURE 3 (A) Changes of 8-epi-$PGF_{2\alpha}$ concentrations (pg ml^{-1}), (B) total glutathione levels (TGSH = GSH + GSSG) (μM) and (C) uric acid concentrations (μmol l^{-1}) in BAL fluid collected before (D_0), and after one (D_1), three (D_3) and seven (D_7) O_3 exposures (0.75 ppm, 12 h day^{-1}). Data are presented as means ± SD. ◆ Significantly different from D_0 values, ♣ significantly different from D_1 values, with $P < 0.05$.

$r = 0.589$) and neutrophil percentage ($P = 0.01$, $r = 0.623$).

With regard to RTLF antioxidant markers, there was no significant correlation between glutathione and functional or inflammatory variables. 8-Epi-$PGF_{2\alpha}$ concentrations were correlated with MaxΔPpl ($P = 0.0184$, $r = 0.487$) and PaO_2 ($P = 0.051$, $r = -0.41$), whereas uric acid was correlated with C_{dyn} ($P = 0.012$, $r = -0.55$), PaO_2 ($P = 0.001$, $r = -0.679$) and neutrophil percentage ($P = 0.0007$, $r = 0.68$). None of the markers were correlated with BAL total protein, and correlation between 8-epi-$PGF_{2\alpha}$, glutathione and uric acid was not significant.

DISCUSSION

In this study, the acute, intermediate and late responses of calves to multiday O_3 exposure have been measured by assessment of pulmonary function, lung inflammation and lung antioxidant status. It was hypothesised that adaptation of pulmonary function was linked to an upregulation of the lung's antioxidant defence. There was no control study on unexposed calves associated. This might limit to some extent interpretation of data provided by repeated BAL because the latter might induce local airway inflammation,[27] even if lung side and lavaged bronchial segments were changed each time BAL was performed. However, in comparison with the intensity of the airway inflammation induced by O_3 exposure, the potential contribution of BAL-induced inflammation should be reasonably reduced.

Pulmonary Function

The significant reductions of C_{dyn} and PaO_2 and increased plasma lactate concentrations (Fig. 1A–C)

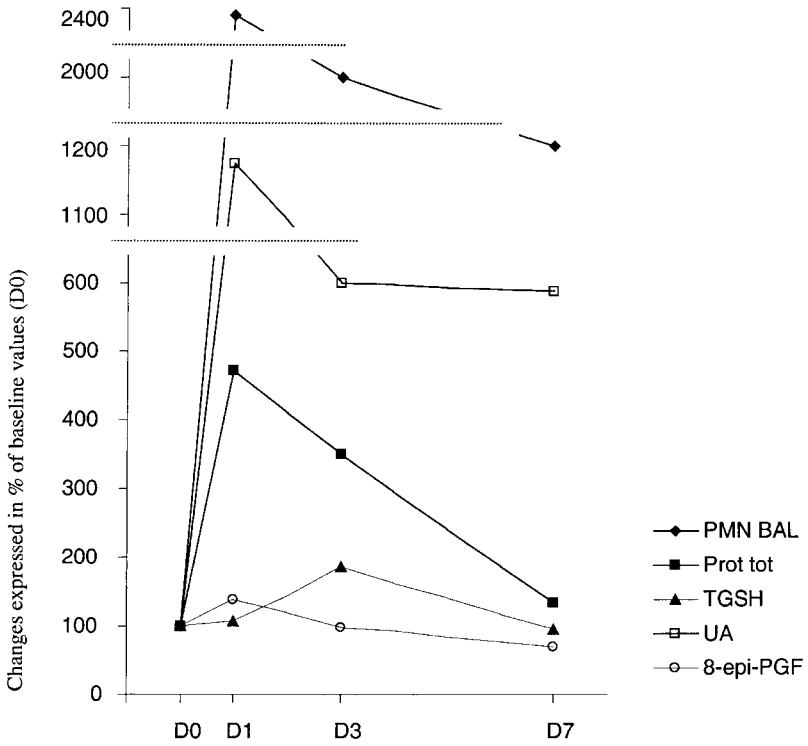

FIGURE 4 Changes of markers of pulmonary inflammation and oxidative stress at Day 1, 3 and 7 of O_3 exposure (0.75 ppm, 12 h day^{-1}) expressed as percentage of baseline values assessed at pre-exposure on Day 0. Data are presented as means, SD were omitted for clarity.

measured after the first O_3 challenge corresponded to the clinically obvious lung oedema. These finding were in accordance with the results of previous studies performed in rabbits and rats,[28,29] in which exposure to high concentration (2–3 ppm) of O_3 induced decreased dynamic compliance, alveolar oedema and hypoxia. In humans, pulmonary oedema has not been reported,[7,21,30,31] probably because of much lower O_3 exposure intensity and duration. The increased lactate concentrations reflected anaerobic muscle metabolism, increased either by reduced oxygen transfer from the lungs to the arterial blood or by elevated oxygen consumption due to increased work of breathing.[32] Lactates have been shown to be a reliable prognostic indicator in calf lung disorders.[22] The lung oedema was present until Day 3, as shown by clinical examination and significantly reduced C_{dyn} and PaO_2. Nevertheless, the progressive raise of C_{dyn} and PaO_2 and normalised lactate levels indicated that lung function impairment was resolving, despite the significant O_3 challenge used in this study. This reduction of lung dysfunction after several O_3 exposures corresponds to the phenomenon termed tolerance or adaptation.[2,3,33]

Lung Inflammation

The O_3 challenge induced a neutrophilic airway inflammation accompanied by cellular damage and epithelial or vascular leakage as quantified by increased concentrations of total protein in BAL (Figs. 2A,B and 4). These observations are in agreement with previous studies demonstrating that acute exposure to O_3 causes an early influx of neutrophils and airway epithelial injury.[21,30,31] In the present study, neutrophil recruitment was important at Day 1. This sharp initial increase was probably due to the exposure duration (12 h) on one hand and to the significant O_3 concentration (0.75 ppm) on the other. Repeating airway endoscopy has been shown to irritate airway epithelium and to induce neurophil influx,[27] which might potentially account for the neutrophil increase. However, by repeating O_3 exposures, neutrophil percentage and total protein concentration decreased progressively, suggesting an attenuation of the inflammatory response and the airway injury despite repeating bronchoscopy and BAL. The neutrophil count reflected the functional adaptation to a lesser extent, whereas total protein modifications underlined this phenomenon. The low pulmonary granulocytic clearance might account for this delayed decrease in neutrophil count.[34]

Antioxidant Response and Oxidative Damage

The first interface encountered by inhaled O_3 is the RTLF.[1] The modifications occurring at this barrier are likely to reflect the early effects of acute and chronic O_3 exposure on the airways and therefore become a hallmark in O_3 studies.

8-Epi-$PGF_{2\alpha}$, glutathione and uric acid were significantly affected by O_3 exposure. BAL 8-epi-$PGF_{2\alpha}$ was significantly increased after the first exposure (Figs. 3A and 4), indicating acute oxidative damage. This was in agreement with the results of Hazbun and co-workers, who reported a significant increase of 8-epi-$PGF_{2\alpha}$ in tracheal lavage of humans after a single O_3 exposure (0.25 ppm, 1 h).[17] On Day 3, 8-epi-$PGF_{2\alpha}$ concentrations were similar to those at Day 0, and at Day 7 levels were even significantly lower than at pre-exposure, suggesting a reduction of phospholipid membrane peroxidations. These findings are supported by those of Jörres and co-workers, who reported an acute increase of malondialdehyde (MAD) concentration in BAL fluid in humans exposed for 4 h to 0.2 ppm O_3, but baseline MDA levels after four days of consecutive O_3 challenge.[21] As BAL 8-epi-$PGF_{2\alpha}$ was neither correlated with C_{dyn} and BAL total protein, nor with BAL neutrophil percentage, it is unlikely that 8-epi-$PGF_{2\alpha}$ increase was due to plasmatic 8-epi-$PGF_{2\alpha}$ transudation or to neutrophilic oxidative burst. An original finding was the significant correlation between BAL 8-epi-$PGF_{2\alpha}$ and PaO_2, which stands in favour of a relationship between pulmonary oxidative damage and lung function.

GSH, GSSG and consequently TGSH and GRR were not significantly modified after the first O_3 exposure, but on Day 3, GSH and GSSG were significantly increased as compared with Day 1. After the seventh exposure, GSH was slightly lower than at Day 0, and GSSG slightly higher (Fig. 3B). Recently, Avissar and co-workers have shown that in humans ELF glutathione peroxidase (Gpx) activity and extracellular Gpx protein levels were significantly decreased for at least 18 h after a single O_3 challenge (0.22 ppm, 4 h).[20] This would imply a reduced GSH oxidation capacity and might explain that we found unchanged GSH and GSSG levels after the first O_3 challenge, which is in disagreement with previous studies reporting significant increases of BAL GSH after single O_3 exposures.[21,35] The increase of BAL glutathione observed at Day 3 was similar to that reported previously in humans exposed to four consecutive O_3 challenges (0.2 ppm, 4 h day^{-1}).[21] Increased glutathione levels have been interpreted as either release of intracellular glutathione due to cellular lysis or export and/or increased synthesis of intracellular glutathione.[17] As the glutathione alterations did not concomitantly occur with the acute pulmonary oedema (C_{dyn} and BAL total protein), the first hypothesis seems less likely. In accordance with the second hypothesis, Boehme and co-workers have shown that GSH content and Gpx activity were increased in BAL cells from rats after three days of O_3 exposure (0.8 ppm, 6 h day^{-1}) and returning to control values after seven days.[36]

Uric acid levels in BAL were significantly increased after the first exposure and decreased only slightly during the following days (Figs. 3C and 4). Intermediate and endpoint levels remained constantly higher than baseline values, suggesting an adaptation with sustained antioxidative defence. These results are in agreement with previous studies, reporting increased uric acid concentrations in BAL of humans after a single exposure,[7,21] but also elevated uric acid concentrations in BAL cells from guinea pigs exposed to O_3 for 1 week.[9] However, normalised uric acid concentrations have been reported after four consecutive O_3 exposures (0.2 ppm, 4 h day^{-1}) in humans,[21] suggesting that the exposure regimen used in this study did not maintain uric acid synthesis to the same extent as in the present report. Our results show for the first time that BAL uric acid concentrations increased rapidly and remained elevated after several exposures. According to Mudway and co-workers,[10] uric acid initially increased to offset O_3-dependent oxidative loss, but the acute response was dampened by repeated exposures. As for the origin of uric acid, it can be speculated either that uric acid movement from the plasma pool to RTLF was enhanced or that the pulmonary synthesis of uric acid was increased.[7] This increase might be a consequence of an upregulated adenosin metabolism and subsequent xanthine oxidase activity due to inflammation.[9] Uric acid was significantly correlated with BAL neutrophils and C_{dyn}, but not with BAL total proteins. These findings stand in favour of inflammation-associated uric acid synthesis, rather than a uric acid influx due to pulmonary oedema.

By comparing the relative changes of each marker, differences in their reactivity to the multiday O_3 challenge were evident (Fig. 4). Interestingly, there was no evidence for acute RTLF antioxidant depletion after the first exposure, despite the intensive O_3 challenge applied. This might be due to the exposure duration, 12 h could be sufficient to induce an acute antioxidant response. Furthermore, ascorbic acid, which is proposed to be the most sensitive RTLF antioxidant marker *in vitro*,[10] was not assessed in the present study. Human studies investigating RTLF ascorbic acid depletion after acute and repeated O_3 exposure are inconsistent, mainly because the moment of RTLF sampling is differing between studies.[7,21,37] However, a single O_3 exposure either decreased BAL ascorbic acid[37] or had no significant effect,[7] 6 or 2 h after the challenge, respectively.

In the present study, uric acid underwent the most important relative changes, followed by glutathione and 8-epi-$PGF_{2\alpha}$. This observation confirmed those of Mudway and co-workers,[10] who demonstrated that RTLF uric acid was more reactive towards O_3 than was glutathione, as shown by a dose and

duration-dependent depletion of uric acid and glutathione in ex vivo RTLF models. 8-Epi-$PGF_{2\alpha}$ was mainly a marker of acute oxidative damage, as shown by the rapid decrease observed after three exposures. The origin of this decrease of oxidative damage despite persisting oxidant stress might be the result of the important and sustained antioxidant protection provided by uric acid. The upregulation of this pulmonary defence pathway preceded pulmonary function improvement, but its role in lung function adaptation remains to be established.

In conclusion, we described in this in vivo animal protocol of multiday O_3 exposure the acute, intermediate and late functional, inflammatory and oxidative lung response to O_3. Our study suggests that functional adaptation occurred even after high concentration and long duration O_3 exposures. The neutrophil-driven inflammation induced by O_3 was marked and progressively resolving. The initial increase in BAL 8-epi-$PGF_{2\alpha}$ levels were indicative of acute oxidative damage which was completely abolished after seven exposures. The BAL antioxidant marker, uric acid, was significantly increased and sustained by repeated exposures. The upregulation of this antioxidative pathway might be implicated in the O_3 tolerance phenomenon.

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